GLUTAMATE RECEPTORS IN THE VENTRAL TEGMENTAL AREA: A POTENTIAL MECHANISM INVOLVED IN LONG TERM POTENTIATION

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ABBREVIATIONS

ACH	acetylcholine
AMPA	amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	analysis of variance
AP5	DL-1-amino-5-phosphonopentanoic acid
ASR	acoustic startle reflex
AP	anterior - posterior
ML	medial - lateral
DV	dorsal - ventral
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CS	conditioned stimulis
UCS	unconditioned stimulis
DA	dopamine
EAA	excitatory amino acid
FPS	fear potential startle response
Glu	glutamate
GABA	gamma amino butyric acid
$GABA_A$	gamma amino butyric acid A
LTP	long term potentiation
LDTg	Laterodorsal Tegmental Nucleus
NMDA	N-methyl-D-asparate
PFC	prefrontal cortex
PnC	caudal pontine reticular nucleus
PPTg	pedunculopontine tegmental nucleus
SN	substantia nigra
VTA	ventral tegmental area
6-OHDA	6 – hydroxydopam

ABSTRACT

In the present study, footshock, which produces a powerful aversive emotional response was used in a Pavlovian conditioning experiment as an unconditioned stimulis (UCS), and was paired with the presentation of a light used as a conditioned stimulis (CS). There is an accumulation of evidence that supports the assertion that dopaminergic (DA) neurons within the ventral tegmental area (VTA) are active in processes that contribute to the amygdala-based circuitry involved in regulating emotionally salient responses. To build upon findings implicating VTA DA, excitatory glutamate (Glu), NMDA and AMPA receptors, were examined with respect to their role in Pavlovian conditioned fear responding. Fear potentiated startle (FPS) was used to assess the effects of intra-VTA infused AP5, and intra–VTA infused CNQX on conditioned fear responding in laboratory rats. The administration of the NMDA receptor antagonist AP5 (at 1.0, 2.5, and 5.0ug doses), blocked the ability of a conditioned stimulus (CS) previously paired with footshock to become conditioned to the UCS. Similarly, administration of the AMPA receptor antagonist CNQX (at 1.0, 2.5, 5.0ug doses), inhibited the ability of the CS to become conditioned to the UCS. The results of this study indicate the VTA is an important site for synaptic modifications associated with fear learning, and that activation of excitatory Glutamatergic receptors in the VTA play a necessary part of the processing underlying fear conditioning. Measures of shock reactivity demonstrated that the infusion of AP5 and CNQX into the VTA did not inhibit baseline startle amplitudes. The administration of AP5 and CNQX did not suppress

the perception of footshock as an aversive stimulus. This study provides further definition to established knowledge surrounding the neural processes whereby neutral environmental cues gain negative emotional salience as occurs in fear conditioning. It was hypothesised that the action of excitatory glutamatergic transmission within the VTA acts on NMDA and AMPA receptors is to assist in the acquisition of Pavlovian conditioned fear, possibly through the same synaptic mechanisms that govern LTP.

1.0 INTRODUCTION

In the presence of an aversive stimulus the experience of fear and anxiety typically produces species typical responses promoted to avoid danger and promote an organism's continued survival (Öhman & Mineka, 2001; Rosen & Schulkin, 1998). The emotional responses promoted by fear and anxiety are performed by that animal innately. A reflexive defence response elicited by a threatening stimulus is considered adaptive because it provides a response when required and then subsides once the threat is removed (Mowrer, 1947; Rosen & Schulkin, 1998). Adaptive fear and other emotional states in general are distinguished from pathological states of fear and anxiety. Pathological emotional responses to threat may often develop through normal adaptive fear and anxiety responses. They are also common features of many pathological disorders that display symptoms characterised by over anxiety and excessive fears. These include psychiatric disorders such as drug induced psychosis (Kokkinidis & Anisman, 1980), schizophrenia (Flack, Laird, & Cavallaro, 1999), and generalised anxiety disorder (Sullivan, Coplan, Kent, & Gorman, 1999) for instance. Pathological disorders such as these are typically so severe and chronic in nature that they impair an individual's ability to function in an adaptive fashion. During adaptive fear states, brain activity is raised and then returns to pre-fear levels upon removal of the threatening stimulus. Conversely, pathological fear states and similar psychiatric disorders are characterised by overactive neural circuitry which works to sensitise an individual to perceiving external threats. As a result of this neural potentiation subsequent pathological

responses become more easily initiated (Rosen & Schulkin, 1998). As a consequence it is suggested that the over activation or sensitisation of fear circuits involved in adaptive fear responding become triggered independently and autonomously by stimuli misperceived as dangerous (Rosen & Schulkin, 1998).

It has been shown that human and animal emotional responses to aversive stimuli produces individual neurochemical physiological, behavioural, and autonomic changes (Blanchard, Yudko, Rodgers, & Blanchard, 1993). Alterations in fear associated physiological arousal include increased plasma corticosteroid release, bradycardia or tachycardia (Iwata & LeDoux, 1988), and altered blood pressure, respiration, startle, and alertness (Rosen & Schulkin, 1998). It is important to define the mechanisms responsible for the role it plays in the production of defensive behaviours, including those that occur in developing psychiatric disorders. This is due to the costly psychosocial, psychological, and biological consequences that arise from emotional regulation (McEwen & Mendelson, 1993). Research has demonstrated the importance of the amygdala through its various roles within the neural functioning involved in mediating and modulating the neurochemical processes governing fear conditioning and fear motivated responding (Davis, 1992; 1997; Fendt & Fanselow, 1999; LeDoux, 1992, 2000).

Research into processes underlying fear conditioning has benefited immeasurably from the utilisation of animal models of fear which have provided

the opportunity to both analyse the behavioural and neurochemical components involved in the fear state. This has been accomplished through the manipulation of certain environmental variables, and neurochemical functioning through drug administration, or the lesioning of areas within the brain that are involved in emotional processing. In order to develop a more comprehensive understanding of the complex neural interactions involved in conditioned fear and its consideration of its role, the amygdala in relation to the acquisition and expression of conditioned fear will be discussed. Within this framework established research involving the VTA and its role in fear conditioning will be highlighted and broadened to incorporate research into LTP and findings from this study implicating the excitatory amino acid (EAA) glutamate (Glu), and drugs that moderate the function of Glu's receptors. The action of Glu NMDA and AMPA receptors will be discussed in relation to the processes involved in the synaptic strengthening underlying Pavolovian fear conditioning, known namely as long term potentiation (LTP). NMDA receptors have been shown to be involved in the neural processes within the amygdala and other brain areas governing LTP. The NMDA and AMPA receptor antagonists produced results that indicate an important role for VTA and the function of its neural circuitry. Discussion of results of this study will be discussed and implications that come from this experiment will be considered concluding this thesis.

1.1 The Fear Response

To ensure human and mammalian survival in general, the motivating state of fear promotes the automatic activation of defence systems composed of inputs from behavioural, cognitive, and physiological components of the central nervous system (Öhman & Mineka, 2001). Animal and humans both quickly learn to fear stimuli that have the potential to endanger their survival. Fear characteristically activates an animal's escape and avoidance strategies which include freezing, escaping, and or defensively attacking (fight or flight) (Öhman & Mineka, 2001). Species typical defences such as these typically occur automatically, however human defence strategies do profit from more sophisticated pre-conscious and conscious processing. Contingencies that exist between certain environmental and physiological stimuli, can for example come to signal potential negative consequences. These consequences, innately anticipated by an organism serve to heighten fear and trigger those defensive responses innately designed to defend an organism and promote its survival. When fear is viewed in a purely functional way, its purpose then is motivated escape or avoidance (Öhman & Mineka, 2001).

1.2 Fear Conditioning – Fear Potentiated Startle Response

Pavlovian conditioning employs aversive classical conditioning techniques which can be used to decipher neural mechanisms responsible for transforming non threatening external stimuli into conditioned stimuli (CS) with negative

emotional salience (LeDoux, 1993). Conditioned stimuli (CS) such as tones, lights, and contexts that have been paired with an aversive stimulus (UCS) such as footshock, then become able independent of the UCS to elicit an array of functional defensive responses (LeDoux, 1993). The CS acquires its aversive properties via repeated presentations with the UCS (LeDoux, 1993). Within this paradigm the fear potential startle response (FPS) is used as it provides a natural measurement of fear. Once rats are conditioned to several pairings of light (CS) and footshock, the mean amplitude of the rats' startle response to an unconditioned stimulus (US) such as a noise or tone typically between 100% greater when the noise is presented together with the light (CS) (Fendt & Fanselow, 1999).

The measure of fear represented by this paradigm is the fear potentiated startle response (FPS). This is derived by taking the difference between the light plus noise and the noise alone trials (Fendt & Fanselow, 1999). The FPS response displays sensitivity to chemicals that have been shown to mediate, moderate, or inactivate an animal's response to fear eliciting stimuli (Fendt & Fanselow, 1999). This allows investigation into the neural mechanisms that are involved in processing fear. The FPS response is operationally defined by an increase in startle amplitude upon presentations of a cue previously paired with shock (Davis, 1986). The value of fear potentiated startle as a measure of fear lies in its replicability and reliability in both human and animal subjects. Since the startle response is instinctive rather than a learned response, its ability to provide

a behavioural indicator (Falls & Davis, 1995; Hitchcock & Davis, 1991; Kim & Davis, 1993). Benefits of using the startle response as a measure of fear include its ability to provide a behavioural indicator that can be manipulated experimentally (Koch, 1999). Subjects develop fear responses that can be conditioned to various stimuli including the context or setting where the CS and the US are presented. This is due to the ease with which conditioned stimuli can become feared after only a few brief presentations (Koch, 1999).

Stimuli conditioned to be feared evoke in animals a number of innate defensive mechanisms considered to reflect the state of pavlovian conditioned fear. These include the behavioural acoustic startle reflex (ASR), and the fear potentiated startle response (FPS) (Fendt & Fanselow, 1999). Experimental manipulation of the neurocircuitry that mediates learned fear can be made to test whether specific brain structures and specific neural processes are involved in fear conditioning during experimental testing (Fendt & Fanselow, 1999). During fear conditioning when a certain structure within the brain is lesioned, the point in the experiment when this lesion was administered determines what the data can comment on. For example, data gathered from testing where drug administration was given prior to the pre-training stage of conditioning, would relate to the target structures function in the acquisition stage of fear conditioning (Fendt & Fanselow, 1999). On the other hand, results that come from a post-conditioning lesion will provide information of that structure's involvement in the expression of conditioned fear (Fendt & Fanselow, 1999).

It can be inferred that the FPS response provides a survival or protective function for animals and humans. For instance, rats have been observed to display an attenuated acoustic startle response when presented with an aversive stimulus (Greba et al., 2000). Research has shown that when rats are presented with a cue that consistently predicts an aversive event, the CS attenuates the startle response (Davis, 1989). The FPS response also displays high sensitivity to drugs involved in the neural processing responsible for fear production (Fendt & Fanselow, 1999). Drugs such as norepinephrine antagonists, dopamine antagonists, opiod antagonists, NMDA associated glycine receptor antagonists, and NMDA antagonists have been shown to block or reduce the FPS response following administration. The anxiolytic effects of these drugs have also been shown to apply in human studies (Fendt & Fanselow, 1999; Davis, 1986; 1993). To date a number of independent studies into the basic processes underlying FPS have developed a picture which suggests that the enhancement of startle within the Pavlovian conditioned fear paradigm involves the amygdaloid complex (Fendt & Fanselow, 1999).

1.3 The Amygdala and Fear Potentiated Startle Response

Electrical stimulation of the amygdala has been shown to increase the amplitude of the startle response (Rosen & Davis, 1988). To confirm the amygdala's role in the activation of the FPS response, Koch and colleagues administered injections of glutamate into the central nucleus of the amygdala. This produced a strong short latency potentiation of the FPS response, whereas

a long latency increase in the FPS response was produced following selective metobotropic glutamate receptor agonist administration to the amygdala (Koch & Ebert, 1993). Experimental research has shown that the basolateral amygdala plays a key role in fear learning, whereas the central nucleus plays a more predominant role than the basolateral amygdala in conditioned fear expression (Campeau, Miserendino, Davis, 1992; Fendt & Fanselow, 1999).

Research that first investigated the amygdala's role in the production of FPS was carried out by Davis and colleagues nearly thirty years ago. Davis showed that the neural circuitry connecting the amygdala and PnC is a necessary link in the conditioned fear potentiating startle response (Fendt & Fanselow, 1999). It was initially shown that the startle response is potentiated upon caudal pontine reticular nucleus (PnC) activation (Fendt & Fanselow, 1999). Amygdala lesion experiments consistently block FPS using a visual (CS) or an auditory CS (Hitchcock & Davis, 1986; Hitchcock & Davis, 1987). Similarly, inactivation of the ventral amygdalofugal pathway which runs directly via the central nucleus of the amygdala to the PnC, blocks the FPS response (Fendt & Fanselow, 1999). The amygdala's role in emotion is not limited to processing conditioned associations, electrolytic lesions of the rat amygdala demonstrated the entire removal of innate defensive and survival behaviours that are expected to be displayed upon presentation of a natural predator, (i.e. a cat) (Rosen et al., 1998). Electrical stimulation of the amygdala on the other hand has been shown to produce species typical behaviours associated with the fear state, including

acoustic startle (Rosen & Davis, 1988), bradycardia (Kapp, Gallagher, Underwood, McNall. & Whitehorn, 1982), corticosterone release (Dunn & Whitener, 1986), and elevated heart rate (Kapp et al., 1982). The amydaloid complex in general is involved in the neural process that underlie the acquisition and expression of conditioned fear.

It has been established that the amygdala is particularly sensitive to stimuli paired with shock (Fendt & Fanselow, 1999). The amygdala lies within the temporal lobes of the brain and consists of five major subdivisions containing a complex array of interconnected nuclei (Amaral, Price, Pitkanen, & Carmichael, 1992). The amygdala's involvement includes contributing to many of the neuromodulatory components that are involved in fear responding (ie. freezing, heart rate changes, hypoalgesia, and potentiated startle (Rosen & Schulkin, 1998). The amygdala receives inputs from sensory stimuli that induce fear, and it also provides a pathway that is involved in activating certain aspects of fear related behaviours required for the organism to respond to and recover from a threat (Rosen et al., 1998). Of the major divisions, the basolateral amygdaloid nuclei and the central amygdaloid nuclei have received most attention.

Lesions of the central, lateral, and basolateral amygdala produce disruption in the expression of physiological fear related behaviours such as fear induced bradycardia, respiratory changes, startle and freezing (Rosen et al., 1998). Conversely, electrical stimulation of the central nucleus of the amygdala

induces bradycardia, freezing, and increases in acoustic startle responding (Rosen et al., 1998). Both the lateral and the basolateral nuclei of the amygdala receive innervations from an array of sensory, auditory, and nociceptive sources (Rosen et al., 1998). There is evidence that the basolateral and lateral nucleus play a significant role in storing aversive measures (Rosen et al., 1998). For example, cells within the lateral nucleus of the amygdala respond to stimuli conditioned to be feared (Rosen et al., 1998). Lateral and basolateral nuclei contain a large concentration of large pyramidal cells which are similar to cortical cells whose role is to integrate incoming information throughout the cortex (Rosen et al., 1998). It has been shown that administration of N-methyl-D-asparate (NMDA) antagonists, which have been shown to block forms of learning when administered into the amygdala, block fear conditioning (Rosen et al., 1998). Administration of DL-2-amino-5-phosphonopentanoic acid (AP5), a glutamatergic NMDA receptor antagonist into the basolateral nucleus of the amygdala blocked the acquisition of fear. This indicates that the processes that occur during fear conditioning are mediated in some way by NMDA receptors within the amygdala.

1.4 Amygdala and Fear Expression

Research that has involved lesions and drug infusions of the amygdala has shown it to be an important mediating structure involved in the acquisition and expression of conditioned emotional behaviour and defensive responding

(Fendt & Fanselow, 1999; Davis, 1992). The amygdala has been demonstrated to be an essential structure involved in the acquisition of fear potentiated startle. Davis and colleagues (1992) administered the NMDA receptor antagonists AP5, AP7 and pertussin toxic directly into the basolateral nucleus of the amygdala. All three of the drugs administered subsequently blocked the acquisition of fear potentiated startle. This is consistent with the assertion that NMDA receptors in the basolateral nucleus of the amygdala play a role in the neural circuitry associated with the induction of fear conditioning (Fendt & Fanselow, 1999).

It has been shown that lesions administered directly into the amygdala prior to fear conditioning blocked the FPS response (Hitchcock & Davis, 1986). In addition, electrolytic lesions administered to the amygdala post fear conditioning have also been shown to block the FPS response. This indicates that the amygdala's role is not limited to activation by fear eliciting stimuli, but is also involved in mediating conditioned fear expression over time (Kim & Davis, 1993).

Consistent with this assertion are results arising from studies employing excitotoxin and electrolytic lesions of the central nucleus of the amygdala in the fear potentiated startle paradigm (Campeau & Davis, 1995; Falls & Davis, 1995; Hitchcock & Davis, 1986, 1987). Electrolytic lesions of the central nucleus were shown to enhance fear motivated behaviour after lesioning to other brain areas that have been shown to mediate the inhibition of fear responding (Melia, Sananes & Davis, 1991). For example, the observed increase in fear potentiated

startle which has been demonstrated post-lesioning of the septal region of the rat brain, is blocked when the central nucleus of the amygdala is subsequently impaired (Melia et al., 1991). It has been suggested that the predominant role of the amygdala in responding to aversive emotional events is in the formation, initiation, and expression of conditioned pavlovian fear responses. Although the amygdala seems to play a large role in functions contributing to FPS, other limbic, and subcortical regions of the mammalian brain are thought to initiate other mediating and or modulating mechanisms within the fear system around which the amygdala is based.

Circuitry extends from within the central nucleus of the amygdala to the elemental startle pathway. This pathway works to mediate fear potentiated startle expression (Fendt et al., 1999). Kim, Campeau, Falls and Davies (1993) found that injections of CNQX, an AMPA receptor antagonist, into the central or basolateral nucleus of the amygdala, successfully blocked the expression of fear potentiated startle. In summary, although the central nucleus does not play a role in the acquisition of conditioned fear, the role it has in the expression of fear has been established (Fanselow & Kim, 1994).

1.5 The Ventral Tegmental Area Neuroanatomical and Neurochemical Connectivity

The VTA has been defined as a few groups of heterogeneous cells positioned on the midline on the base of the mesencephalon, dorsal to the substantia nigra, however very few clear physical boundaries differentiate the two

structures (Oades & Halliday, 1987). Both areas display similar cellular structure and particularly pathways with innervations. For this reason, dissociation of one area from the other is benefited by functional over structural change. Forebrain projections of DA containing neurons within the A_{10} cell grouping of the Ventral Tegmental Area (VTA) are part of the mesocorticolimbic system. This region is known as the Ventral Tegmental Area (VTA). There is evidence accumulating that infers the VTA plays a prominent role in governing response to reward (Blackburn, Pfaus, & Phillips, 1992; Dahlstrom & Fuxe, 1964; Oades & Halliday, 1987; Salamone 1994; Swanson, 1982; Greba, Munro, & Kokkinidis, 2000). Additional evidence points to the role of the mesoamygdaloid pathway within this system and that it contributes to conditional fear processing (Greba et al., 2000). Swanson (1982) reported that of the approximately 27,000 cells which make up the VTA, it was estimated that up to 70% were dopaminergic. Deutch (1985) and colleagues have demonstrated that activation of VTA DA neurons increases the metabolism of DA within both the central and basolateral nuclei of the amygdala (Coco, Kuhn, Ely, & Kilts, 1992; Deutch, Tam, & Roth, 1985). The two amygdaloid nuclei have shown themselves to be essential in the processing and performance of responses to conditioned fear stimuli (Davis, 1992; Munro & Kokkinidis, 1997). It is suggested from these studies that VTA DAergic transmission within the VTA is involved in the fear circuitry within the mesocorticolimbic system.

1.6 Long Term Potentiation

Long term potentiation was first described by Bliss and Lomo (1973) over thirty years ago. They postulated that neurocircuitry governing learning and memory was mediated by excitatory synaptic changes (Bliss et al., 1973). The hypothesis asserts that long-lasting alterations in synaptic strength occur during learning that enables new associations to be made and stored in memory for later use (Martin, Grimwood, & Morris, 2000). LTP has been demonstrated in the hippocampal formation in many experiments (Stanton & Senowswki, 1989; Christofi, Nowicky, Bolsover, & Bindman, 1993). Since it can be produced in slices of the hippocampal formation held in isolation and also in living animals, LTP provides researchers a model of learning that can be manipulated in order to analyse the effects of biochemical alterations on memory formation (Holscher, 1997).

1.7 Excitatory Glutamate Receptors

LTP is mediated by two cellular processes at the synaptic level. It has been shown that both of these involve the action of the excitatory neurotransmitter glutamate, and the ionotrophic glutamate receptors NMDA and AMPA. When glutamate binds with AMPA receptors, an excitatory post-synaptic potential (EPSP) is produced (Huettner et al., 2003). NMDA receptors contribute to synaptic strengthening through the action of calcium dependent ion channels. Glutamate stimulation of NMDA receptors and post synaptic depolarisation are

both required to initiate LTP. NMDA receptors remain blocked until the post synaptic membrane where the receptor is located is depolarised which promotes the release of Magnesium ions lying within the receptors structure. The action NMDA receptor activation and the depolarisation of the post synaptic membrane promote the entry of Calcium ions which promote a number of intra-cellular processes involved in LTP.

1.8 Ventral Tegmental Area Dopamine and Fear Conditioning

The role of dopamine in the production of conditioned fear has been postulated to involve a number of mesocorticolimbic DA projections that originate in the VTA. Areas within the forebrain that receive VTA DA connections include the amygdala, hypothalamus, hippocampus, and the medial prefrontal cortex. The basolateral amygdala receives VTA DA innervations that have been suggested to mediate emotional learning and memory recall (Greba, Giftkins, & Kokkinidis, 2001; Greba & Kokkinidis 2002; Nader, Schafe, & LeDoux, 2000). DA projections from the VTA have been shown to be involved in motivational behaviour linked to drug, appetite and reproductive reinforcement (Borowski & Kokkinidis, 1996). The VTA comprises an important position within the neuroanatomical system that research suggests plays an essential role in natural reinforcement and the conditional effects of drug abuse (Bonci & Malenka, 1999).

It has been suggested that DA neurons within the VTA may play a role in various processes involved in aversive emotional experiences. It has been shown for example that mild stress exposure to cues conditioned to be feared increases early gene expression and enhances the metabolism of VTA DA (Beck & Fibiger, 1995; Borowski & Kokkinidis, 1996; Deutch, Tam, & Roth, 1985). Deutch and colleagues (1991) demonstrated increases in VTA c-fos elicited by conditioned fear cues. Electrical stimulation of the VTA in cats provokes characteristic fear like behaviours (Stevens & Livermore, 1978). Conversely, VTA lesions in rats produces hypoemotivity (Le Moal, Stinus, & Cardo, 1969). Research that adds further weight to VTA DA's role in conditioned fear expression comes from studies examining the stimulation of the PPTg. Electrical stimulation of PPTg neurons increases DA neural activity within the VTA (Kelland, Freeman, Rubin, Chiodo, 1993; Greba et al., 2000). This is consistent with results found suggesting that PPTg excitation through fear evoking stimuli contributes to emotional disturbances within the amygdaloid complex, and further supports research demonstrating the VTA's role in amygdala mediated DA dependent fear responding (Greba et al., 2000).

Two sources of neurotransmission that regulate DA neural excitation within the ventral mesencephalon include Acetylcholine (ACh) and gamma-amino-butyric-acid (GABA). The release of Ach within the VTA produces an excitatory effect on DA activity (Blaha, Allen, Das, Inlis, Latimer, Vincet, & Winn,

1996; Hallanger & Wainer, 1988; Jackson & Crossman, 1983; Oakman, Faris, Kerr, Cozzari, & Hartman, 1995; Woolf, 1991). GABA containing neurons found in the VTA are known to synapse on DA cells and inhibit GABA neural activity (Giftkins, Greba, & Kokkinidis, 2002). It has been shown that fear motivated responding and patterns of DA neural firing are mediated by inhibitory GABA interneurons. The release of Ach within the VTA produces an excitatory function, increasing VTA DA neural activity (Greba et al., 2000). Research has shown that application of Ach, muscarine and carbachol produces increases in DA cell firing. The increase in cell firing, or depolarisation produced by Ach administration is blocked by methylscopolomine, a non-specific muscarinic receptor antagonist (Lacey, Calabresi, North, 1990; Westerink, Kwint, De Vries, 1996; Greba et al., 2000).

A further role for VTA DA arises from its mesolimbic projections to the basolateral and central nuclei within the amygdala (Borowski & Kokkinidis, 1996). Electrical stimulation of the VTA has been shown to potentiate amygdala kindling which contributes to alterations in VTA neural firing (Maeda & Mogenson, 1981). Animal experiments using both chemical and electrical lesions have demonstrated that the effective interference on VTA DA contributes to a disruption in classically conditioned fear (Borowski & Kokkinidis, 1996; Munro & Kokkinidis, 1997). It has also been shown that neurotoxic lesions made within the VTA removes the ability of an externally explicit CS to attenuate the

measurement of the acoustic startle response (Borowski & Kokkinidis, 1996; Davis, 1992; Greba, Munro, & Kokkinidis, 2000).

In addition to DA involvement within the VTA, DA efferents that arise through VTA neurons are projected to the amygdala via the mesocorticolimbic pathway (Greba et al., 2000; Munro et al., 1987; Oades & Halliday, 1987; Swanson, 1982). It has been shown that 6-OHDA lesions of the VTA results in a consistent loss of up to 90% of amygdaloid DA (Oades et al., 1987). The importance of the mesoamygdaloid DA pathway is further strengthened by studies examining the prefrontal cortex and its role in fear responding. For instance, an increase in DA is displayed in both the VTA and the PFC after exposure to a mild stressor (Deutch et al., 1965; Borowski et al., 1996). The action of DA within the PFC is reduced when the central amygdala is lesioned (Borowski et al., 1996), and concentrations within the VTA are lowered as a consequence of lesioning. Within the VTA two major cell types predominate Dopaminergic, and GABAergic. DA neurons are noted to play the primary role, releasing DA via efferents to areas including the nucleus acumbens, amygdala, and prefrontal cortex (Bonci et al., 1999; Swanson, 1982; Munro et al., 1997). It has been shown that the majority of non-DA cells within the VTA are GABAergic and their role is to inhibit the potential of VTA DA neurons (Bonci et al., 1999; Kalivas, 1993; White, Hu, & Henry, 1993). Inputs via from the prefrontal cortex make excitatory synaptic connections onto VTA DA and GABA cells (Bonci et al., 1999; Kalivas, 1993; White et al., 1993) which contain NMDA and non-NMDA

Glu receptors. These glutamatergic receptors work to facilitate synaptic action and also long term potentiation (Bonci et al., 1999).

The $GABA_A$ agonist muscimol, when infused into the VTA suppresses DA neural activity. Muscimol also works to block the expression of FPS when administered to the VTA (Munro & Kokkinidis, 1997). It has been suggested that GABA receptors in the VTA may play a role in the anti-anxiety effects of benzodiazepine drugs which facilitate inhibition by facilitate GABA and chloride channels at the $GABA_A$ complex (Richards & Mohler, 1984). Cholinergic projections from neurons that arise from within the Pedunculopontine Tegmental Nucleus (PPTg) and the Laterodorsal Tegmental Nucleus (LDTg) both innervate the VTA (Blaha et al., 1996; Greba, Munro, & Kokkinidis, 2000). Interestingly, PPTg neurons provide one possible source for EAA projections to the VTA. This raised the possibility that another neurotransmitter, glutamate, may be involved in the synaptic neural plasticity associated with aversive emotional function of fear conditioning. It has been demonstrated that VTA DA neuronal activity increases upon administration of both glutamate and NMDA to midbrain VTA slices (Wang & French, 1993). NMDA administrated directly into the VTA produces an increase in locomotor activity (Kalivas, Duffy, Barrow, 1989; Pycock & Dawbarn, 1980; Willick & Kokkinidis, 1995). Conversely, in vivo electrophysiological experiments have demonstrated that DA and DA agonist drug administration to the VTA produces a reduction in DA neuronal firing rates (White & Wang, 1984).

It has been demonstrated that mesolimbic DA neurons are regulated by somatodendritic DA D_2 receptors (White et al., 1997). Administration of the DA $D_{2/3}$ agonist quinpirole to in vitro VTA slice preparation inhibits VTA DA neurons (Munro & Kokkinidis, 1997). It does this through its actions on DA autoreceptors, therefore a decrease in VTA DA is most likely the reason for quinpirole's suppression of fear potentiated startle (Borowski & Kokkinidis, 1996).

2.0 MATERIAL and METHOD

2.1 Subjects

Fifty six naïve male albino rats of the Wistar strain were used for the experiments. The rats weighed approximately between 300-350g at the beginning of the experiments. The rats were bred in the University of Canterbury's Psychology laboratory and were held in a climatically controlled colony environment, in grouped-housing with free access to food and water. Animals were maintained on a 12 hour light and dark cycle (lights on at 8am) and behavioural testing was conducted during the light cycle.

2.2 Apparatus

The acoustic startle reflex amplitudes and footshock delivery was measured in four identical cages (16.5 cm x 8 cm x 9 cm) located inside sound attenuating melamine chambers (60 cm x 34 cm x 56 cm). The sides and lid of

each startle cage (Med Associates, Fairfield, VT) were manufactured from stainless steel horizontal rods 0.25 cm in diameter and were situated 1.5 cm apart. The floor of the startle cage also consisted of stainless steel rods but with a 0.45 cm diameter. A metal frame 10 cm away from each cage housed a 6.0 cm speaker. The startle cages were mounted on a Med Associates load cell-based startle platform (25 cm x 11.5 cm x 4.5 cm). Movement amplitude was rectified, digitised, and recorded by Med Associates software which controlled the white noise and scrambled shock stimuli. The acoustic stimulus produced by a programmable audio generator consisted of a 100-ms white noise burst with a rise-decay time of 10 ms. Ambient noise level in each chamber was 36 dB as measured by a Bruel & Kjaer (Model 2235; Denmark) sound level meter (A Scale). The 600-μA footshock was delivered through the floor grid by constant current stimulators connected to commutators located on top of each sound attenuating chamber with stimulation leads attached to each startle cage.

2.3 Procedure

2.3.1 Surgery

Surgery was performed in accordance to the animal protocols that was approved by the Animal Ethics Committee at the University of Canterbury. Subjects first received Atropine Sulphate (0.12 mg/kg) prior to anaesthetic in order to dry-up mucous secretions. Twenty minutes later they were anaesthetised with Sodium Pentobarbitone (90 mg/kg) and put in a Stoelting Stereotaxic instrument (WoodDale, IL). Subjects also received a Mepivacaine

(local anaesthetic) scalp injection (20 mg/ml) before surgery and Ketofen, an anti-inflammatory (10 mg/ml) local injection prior to suturing as required by the ethical guidelines. The horizontal plane of the subject's skull was levelled using the landmarks Bregma (anterior) and Lambda (posterior). Stereotaxic co-ordinates were then calculated by using the stereotaxic atlas of the rat brain by Paxinos and Watson (1986; 1998). Stainless Steel guide cannulas (C313G, Plastics One, Roanoke, VA) were implanted bilaterally with an outer diameter of 0.71mm. The co-ordinates for bilateral implants were at a 10° angle aimed 1.0mm above the medial VTA, AP - 4.8mm, ML ± 2.4mm and DV – 7.5mm. The implants were then fixed to the skull using dental cement and four stainless steel jeweller's screws (Lomat, Quebec, Canada). Subjects were then left to recover for approximately seven days before commencement of testing.

2.3.2 Baseline Acoustic Startle (Pre-drug)

The procedure of the proposed experiment has been approved by the Animal Ethics Committee of the University of Canterbury. Seven days post-surgery each subject's acoustic startle threshold was measured. The rat was placed into the startle apparatus and given a 5-min period of acclimatisation. They then received two sessions of 30 white noise bursts with a fixed interval of 20-s between each noise burst. The decibel level of the white noise bursts alternated between 91, 95 and 99 in intensity. Each subject were assigned to a particular decibel level according to their threshold.

2.3.3 Pavlovian Conditioning

The rats were put through a two day testing procedure consisting of Pavlovian conditioning and a fear test. Day one comprised of drug infusion prior to Pavlovian conditioning in which the subject was taken out of its holding cage and infused with either AP5, CNQX, or saline. The dummy cannulas were removed (C313DC, Plastics One) and 28-gauge (0.36) stainless steel infusion needles (C313I, Plastics One) were then inserted into each cannula. Polyethylene tubing (PE20, Plastics One) was pre-loaded with either AP5, CNQX or saline and attached to each cannula implant of the subject. The polyethylene tubing was attached to a 2µl Hamilton syringe and infused over a 1 minute period at a volume of 0.5 µl per side with infusion pumps (Model 310, Stoelting). After infusion stops, the needles were then left for a further 2 minutes, and then removed and replaced with the dummy cannulas. Pavlovian fear conditioning consisted of 20 pairings of light + shock which was presented to the subject in the startle chamber. The light (CS) was presented for a duration of 3.5-s, immediately followed by a 500-ms scrambled footshock (600µA) with an inter-stimulus interval of 56-s. The second day consisted of a fear-potentiated startle test to measure the subject's fear response. The test involved 10 white-noise bursts set at the subject's baseline startle level, with an inter-stimulus interval of 30-s followed by 5 noise alone test trials and 5 noise + light trials, with an inter-stimulus interval of 30-s. The light (CS) was presented for 3.5-s, followed immediately by a 100ms noise burst.

2.4 Perfusion and Histology

Fifty subjects with bi-cannula implants into the VTA were culled and then perfused intercardially with saline, followed by a 10% formalin solution. The brains were then removed quickly and stored in the formalin solution for one-day, and later transferred to a sucrose solution and refrigerated. The subjects' brains were then sliced (50µm) after 2-3weeks storage using the cryostat and then mounted onto gel coated slides. The slides were then stained with cresyl violet and later evaluated using a microscope and the Stereotaxic atlas of the rat brain by Paxinos and Watson (1986; 1998) in order to verify the guide cannula placements.

2.5 Statistical Analysis

The results of the expression of Pavlovian conditioned fear were analysed by a One-Way Analysis of Variance (ANOVA) in order to detect any significant differences in the acoustic startle levels between the experimental and control groups. To assess shock reactivity, movement scores were analysed by a One-Way ANOVA. Movement scores were evaluated to observe if the drug had any effect on the animal's ability to react to the shock and light in the Pavlovian Conditioning sessions.

3.0 RESULTS

3.1 AP5

Histology

Three rats in the AP5 5ug and 2.5ug were excluded from the study after developing loose acrylic headcaps. Guide cannula locations in the VTA for the remaining rats in each drug treatment group (AP5 5ug, N= 7, AP5 2.5ug, N=6, AP5 1.0ug, N= 8) are depicted in Figure 1.

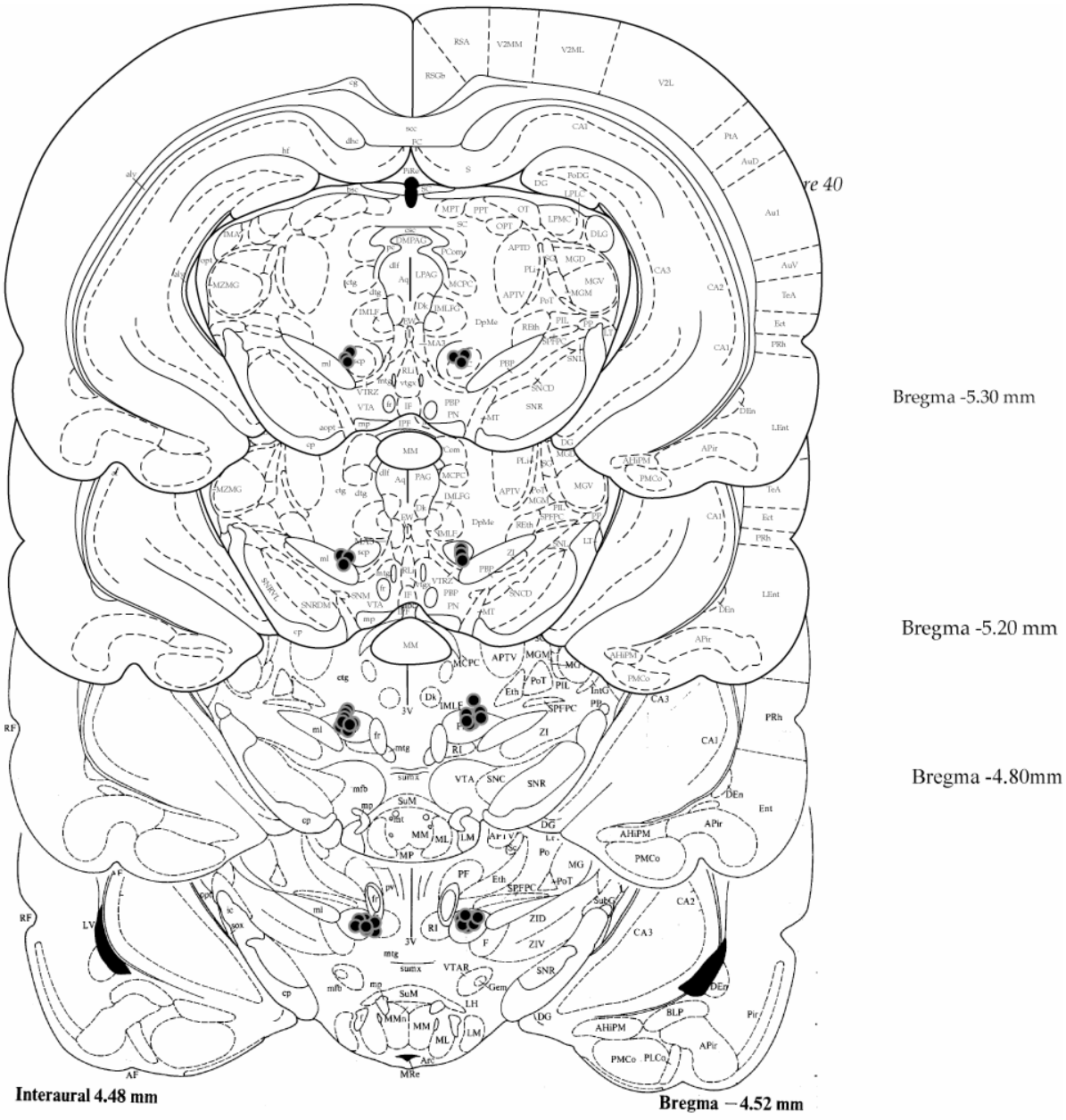

Figure 1. A schematic representation of the guide cannula locations for the AP5 5ug (N = 7), AP5 2.5ug (N = 6), and AP5 1.0ug (N = 8) infusion groups. Guide cannual placements were implanted at a 10° angle aimed 1mm above the VTA (co-ordinates AP - 4.8mm from bregma, ML ± 2.4mm from the sagital suture, DV-7.5mm from the skull surface). The representative sections (- 4.52mm, - 4.80mm, - 5.20mm, and - .30mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by Paxinos and Watson, 1986 and 1998.

3.2 Acoustic Startle and Selected Decibel Levels

The acoustic startle levels of the individual animals did not differ significantly across the Saline and AP5 groups during the baseline screening session, $F(3,25) = 0.34$, $p = 0.80$. Therefore, any difference seen in the startle levels of the animals post pavlovian conditioning were not as a result of variable baseline levels. The decibel levels selected for each subject also did not differ significantly between the Saline and AP5 groups, $F = (3,25) = 0.45$, $p = 0.72$. The noise burst decibel level varied between 91 – 99 dB, with an average of 94.59.

3.3 Effect of AP5 Drug Infusion into the VTA on the Expression of Pavlovian Conditioned Fear

In this experiment the Pavlovian conditioning sessions of 20 light + footshock pairings was accompanied 24 hours later by a fear potentiated startle test. The behavioural data are shown in Figure 2. Subjects that received intracranial infusions of saline served as a control group (N=8) for the animals that received intracranial infusions of AP5 prior to Pavlovian Conditioning sessions. A one-way ANOVA of the difference scores (light + noise – noise alone startle scores) was carried out between the AP5 (experimental) and saline (control) groups. ANOVA of the difference scores revealed a significant main effect for drug treatment, $F(1,13) = 22.41$, $p<0.0004$ (AP5 5ug vs. Saline), $F(1,12) = 6.66$, $p<0.024$ (AP5 2.5ug vs. Saline), and $F(1,14) = 11.97$, $p<0.004$ (AP5 1.0ug vs. Saline) represented in Figure 3. The results reveal that infusions

of AP5 5ug, 2.5ug and 1.0ug into the VTA significantly decreases the startle amplitudes of the animals in response to the light + noise presentations. Post-hoc analysis using the Bonferroni test confirmed that the three varying doses of AP5 used in the current study significantly decreased the fear potentiated startle response relative to the saline-treated control group. Therefore, the subjects in the three varying drug groups failed to demonstrate Pavlovian fear conditioning after receiving intra-VTA drug infusions of AP5 followed by 20 light + footshock pairings in comparison to the control group.

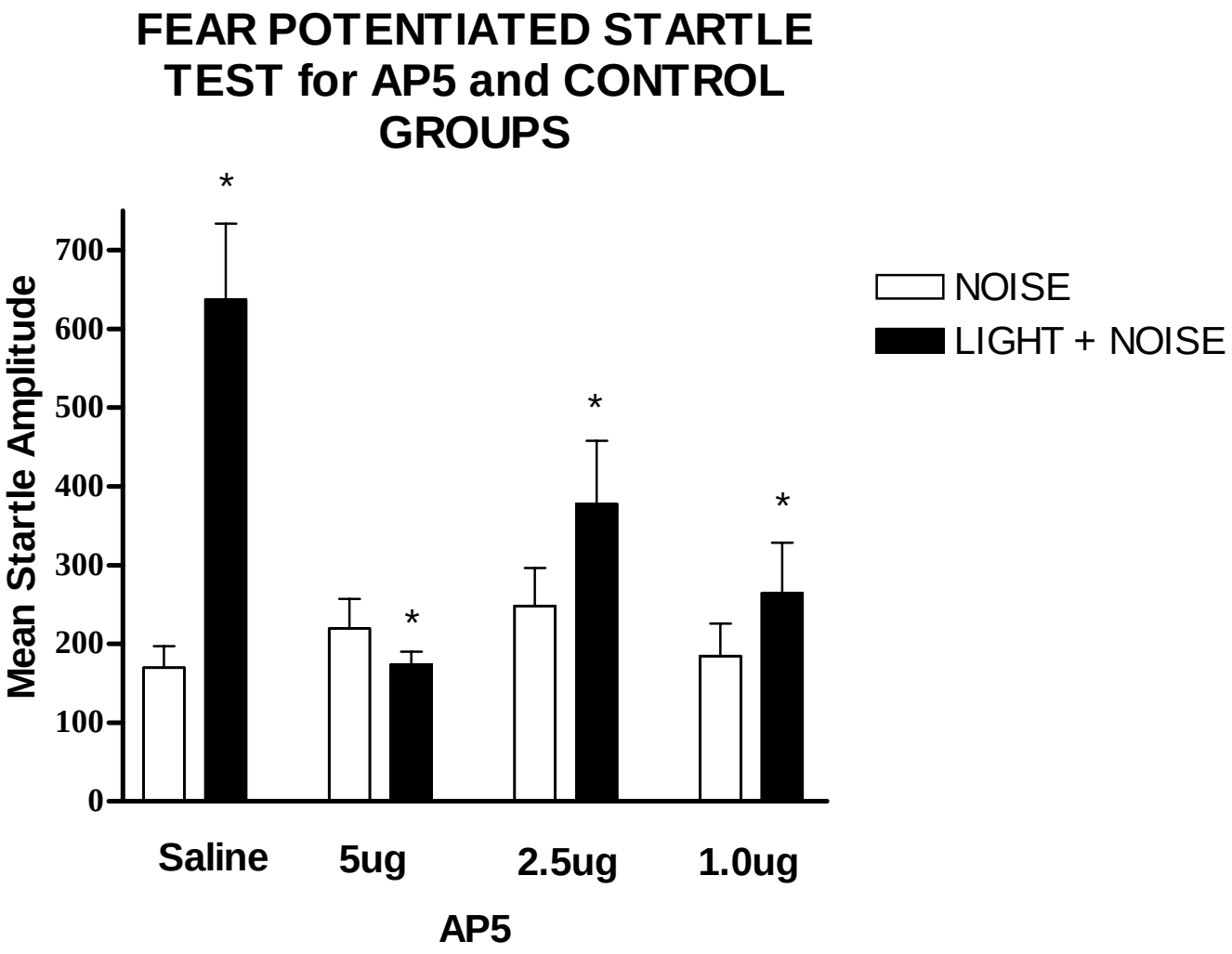

Figure 2. ANOVA results for subjects infused with saline (N = 8) or AP5 5ug (N = 7), AP5 2.5ug (N = 6), and AP5 1.0ug (N = 8) into the VTA. Animals were Pavlovian Fear Conditioned and tested for fear-potentiated startle 24h following 20 sessions of light + footshock. The AP5 drug groups significantly failed to show a light-associated increase of acoustic startle compared to the saline group (* $p<0.0004$, $p<0.02$, $p<0.004$).

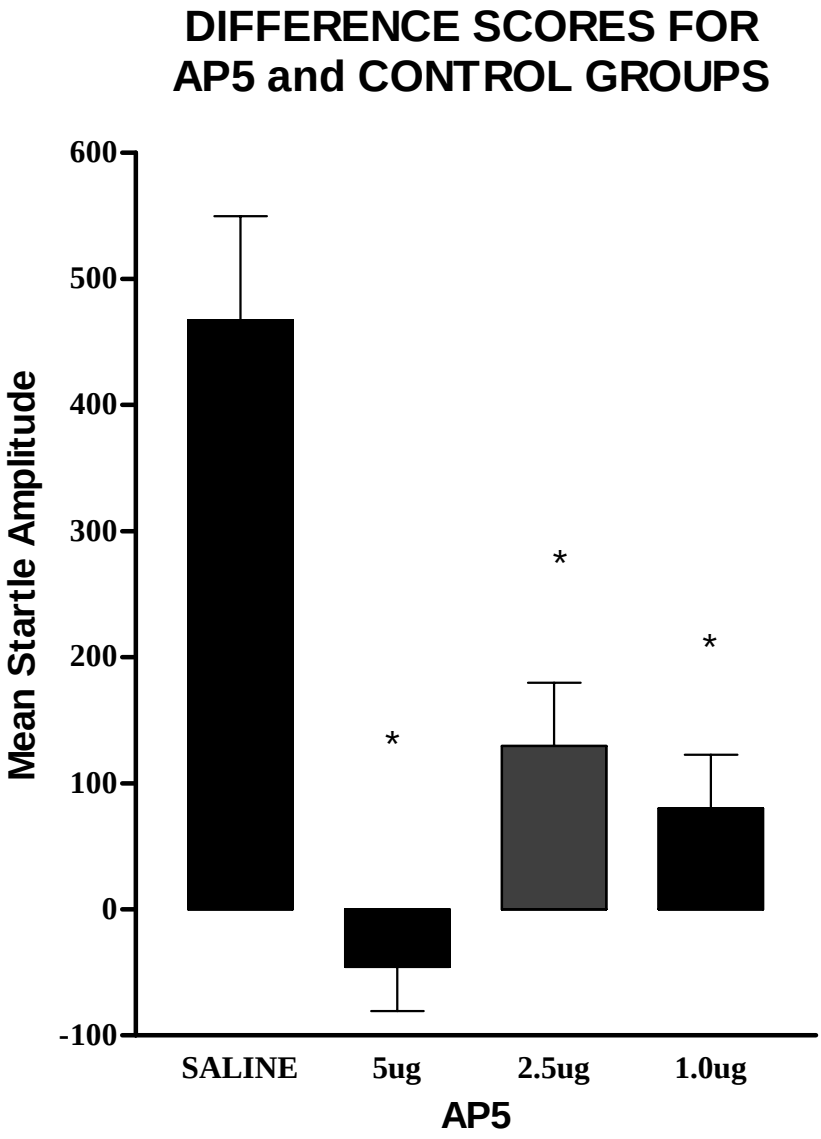

Figure 3. Mean difference scores (light + noise – noise alone) following infusion of saline (N = 8) or AP5 5ug (N = 7), AP5 2.5ug (N = 6), and AP5 1.0ug (N = 8) into the VTA.

3.4 Shock Reactivity

In order to demonstrate that drug infusions did not significantly attenuate the shock reactivity of the subjects a one-way ANOVA was used. There was no significant difference in shock reactivity between the AP5 drug group and the saline group,

$F(2,26) = 1.47$, $p<0.25$, ns. Therefore, the anxiolytic actions of AP5 infused into the VTA cannot be effected by possible drug effects on sensorimotor responding as a suppression of movement amplitude was not induced by the AP5 infusion (see Figure 4).

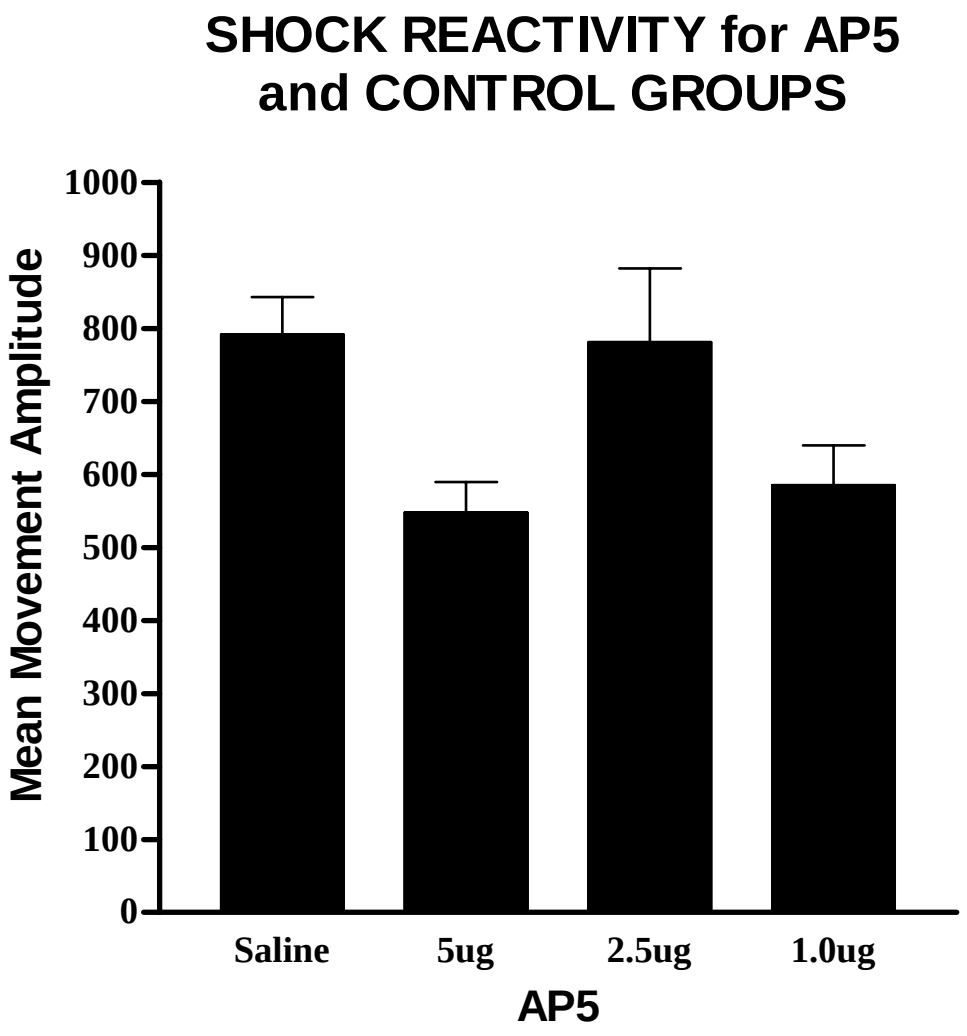

Figure 4. Mean movement amplitude recorded 100ms before shock and 100ms after shock onset following infusion of saline (N = 8) or AP5 5ug (N = 7), AP5 2.5ug (N = 6), and AP5 1.0ug (N = 8) into the VTA. ANOVA did not reveal any significant difference between the groups.

4.0 RESULTS

4.1 CNQX

Histology

Three rats in the CNQX 2.5ug and 1.0ug were excluded from the study after developing loose acrylic headcaps. Guide cannula locations in the VTA for the remaining rats in each drug treatment group (CNQX 5ug, N= 8, CNQX 2.5ug, N=6, CNQX 1.0ug, N= 7) are depicted in Figure 5.

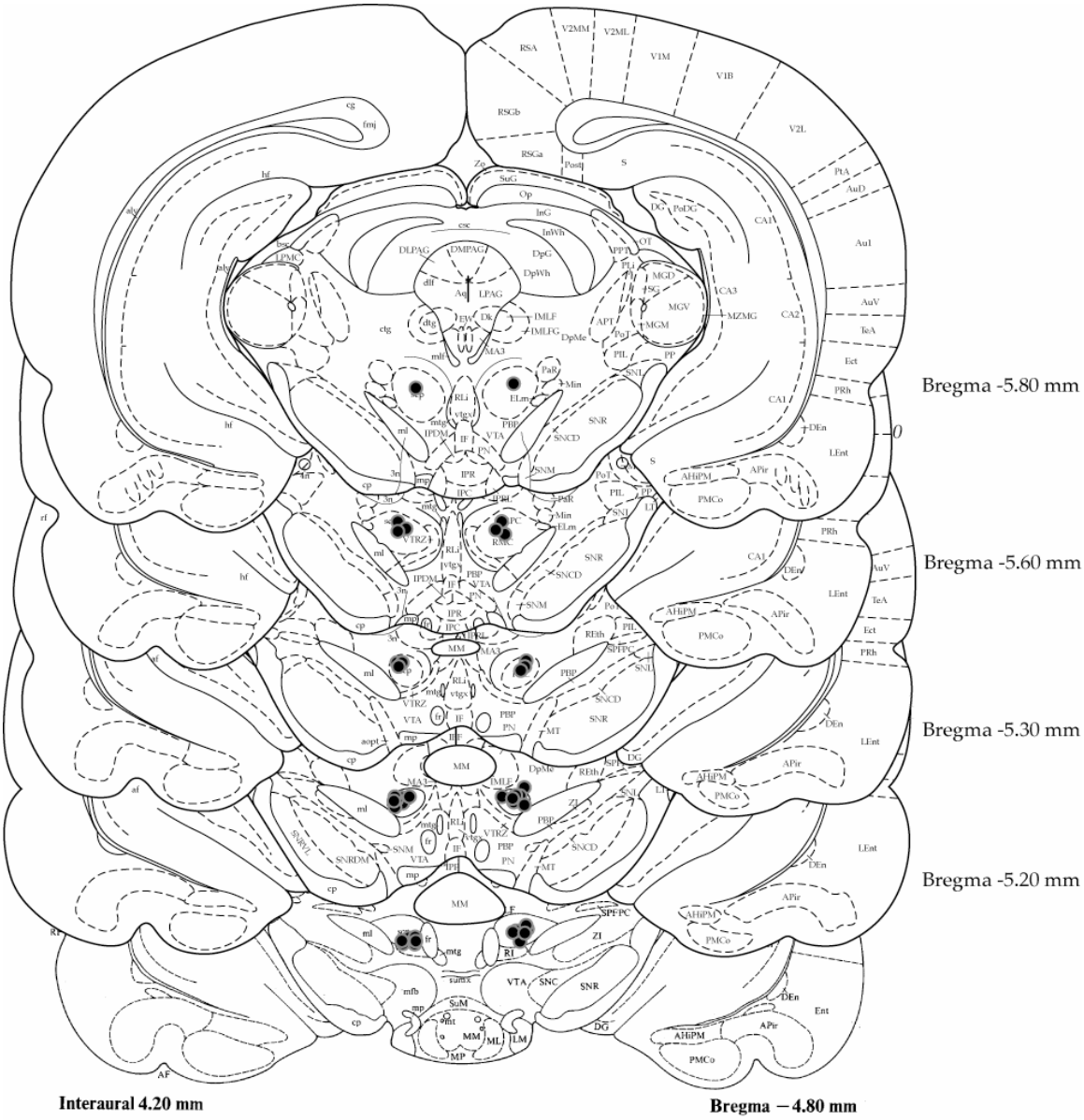

Figure 5. A schematic representation of the guide cannula locations for the CNQX 5ug (N = 8), CNQX 2.5ug (N = 6), and CNQX 1.0ug (N = 7) infusion groups. Guide cannual placements were implanted at a 10° angle aimed 1mm above the VTA (co-ordinates AP - 4.8mm from bregma, ML ± 2.4mm from the sagital suture, DV- 7.5mm from the skull surface). The representative sections (- 4.80mm, - 5.20mm, - 5.30mm, - 5.60mm, and - 5.80mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by Paxinos and Watson, 1986, 1989

4.2 Acoustic Startle and Selected Decibel Levels

The acoustic startle levels of the individual animals did not differ significantly across the Saline and CNQX groups during the baseline screening session, $F(3,25) = 0.66$, $p = 0.59$. Therefore, any difference seen in the startle levels of the animals post pavlovian conditioning were not as a result of variable baseline levels. The decibel levels selected for each subject also did not differ significantly between the Saline and CNQX groups, $F = (3,25) = 0.61$, $p = 0.61$. The noise burst decibel level varied between 91 – 99 dB, with an average of 94.72.

4.3 Effect of CNXQ Drug Infusion into the VTA on the Expression of Pavlovian Conditioned Fear

In this experiment the Pavlovian conditioning sessions of 20 light + footshock pairings was accompanied 24 hours later by a fear potentiated startle test. The behavioural data are shown in Figure 6. Subjects that received intracranial infusions of saline served as a control group (N=8) for the animals that received intracranial infusions of CNQX prior to Pavlovian Conditioning sessions. A one-way ANOVA of the difference scores (light + noise – noise alone startle scores) was carried out between the CNQX (experimental) and saline (control) groups. ANOVA of the difference scores revealed a significant main effect for drug treatment, $F(1,14) = 13.1$, $p<0.003$ (CNQX 5ug Vs. Saline), $F(1,12) = 12.2$, $p<0.004$ (CNQX 2.5ug Vs. Saline), and $F(1,13) = 9.2$, $p<0.01$

(CNQX 1.0ug Vs. Saline) represented in Figure 7. The results reveal that infusions of CNQX 5ug, 2.5ug and 1.0ug into the VTA significantly decrease the startle amplitudes of the animals in response to the light + noise presentations. Post-hoc analysis using the Bonferroni test confirmed that the three varying doses of CNQX used in the current study significantly decreased the fear potentiated startle response relative to the saline-treated control group. Therefore, the subjects in the three varying drug groups failed to demonstrate Pavlovian fear conditioning after receiving intra-VTA drug infusions of CNQX followed by 20 light + footshock pairings in comparison to the control group.

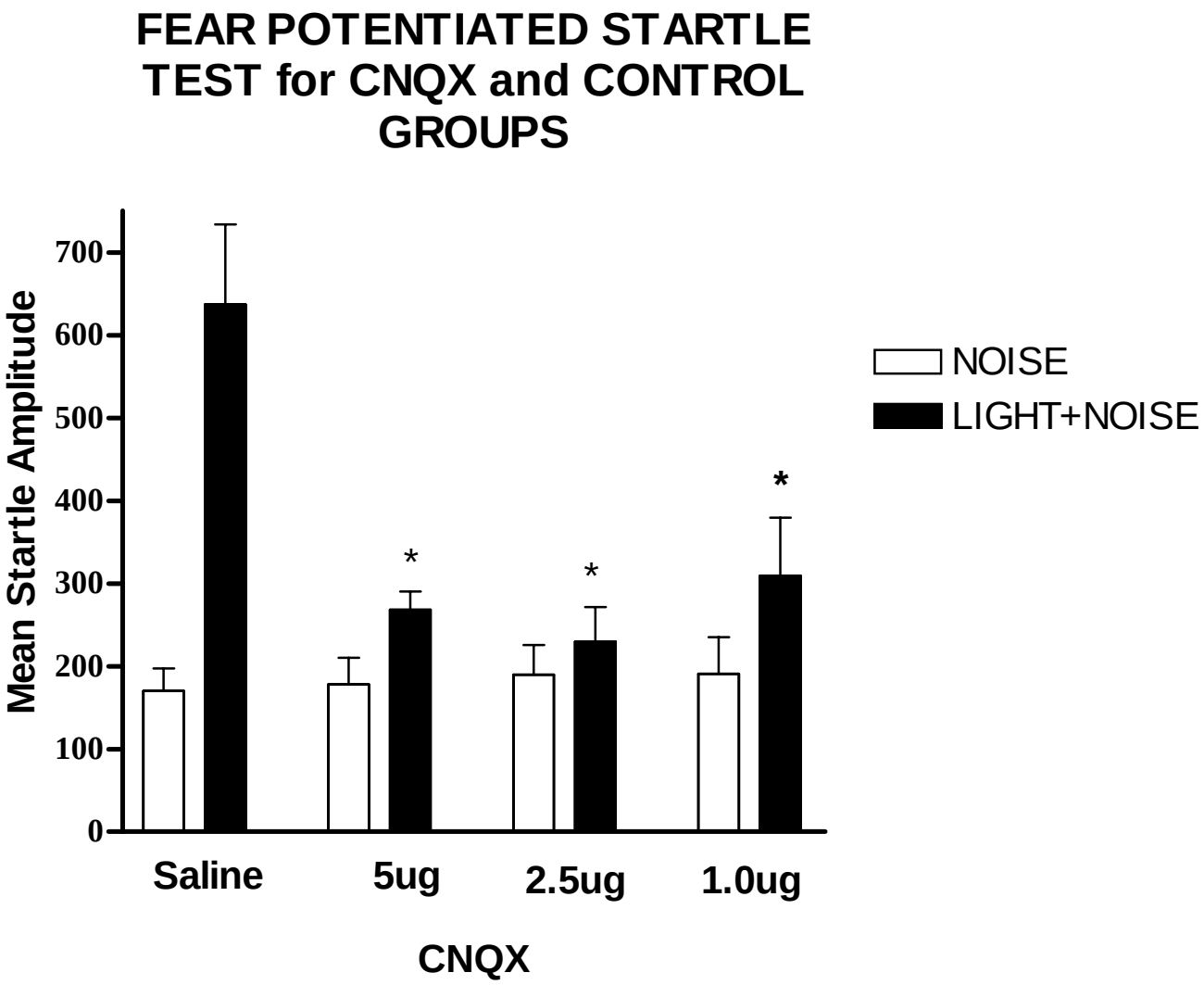

Figure 6. ANOVA results for subjects infused with saline (N = 8) or CNQX 5ug (N= 8), CNQX 2.5ug (N=6), CNQX 1.0ug (N= 7) into the VTA. Animals were Pavlovian Fear Conditioned and tested for fear-potentiated startle 24h following 20 sessions of light + footshock. The CNQX drug groups significantly failed to show a light-associated increase of acoustic startle compared to the saline group (* $p<0.003$, $p<0.004$, $p<0.01$).

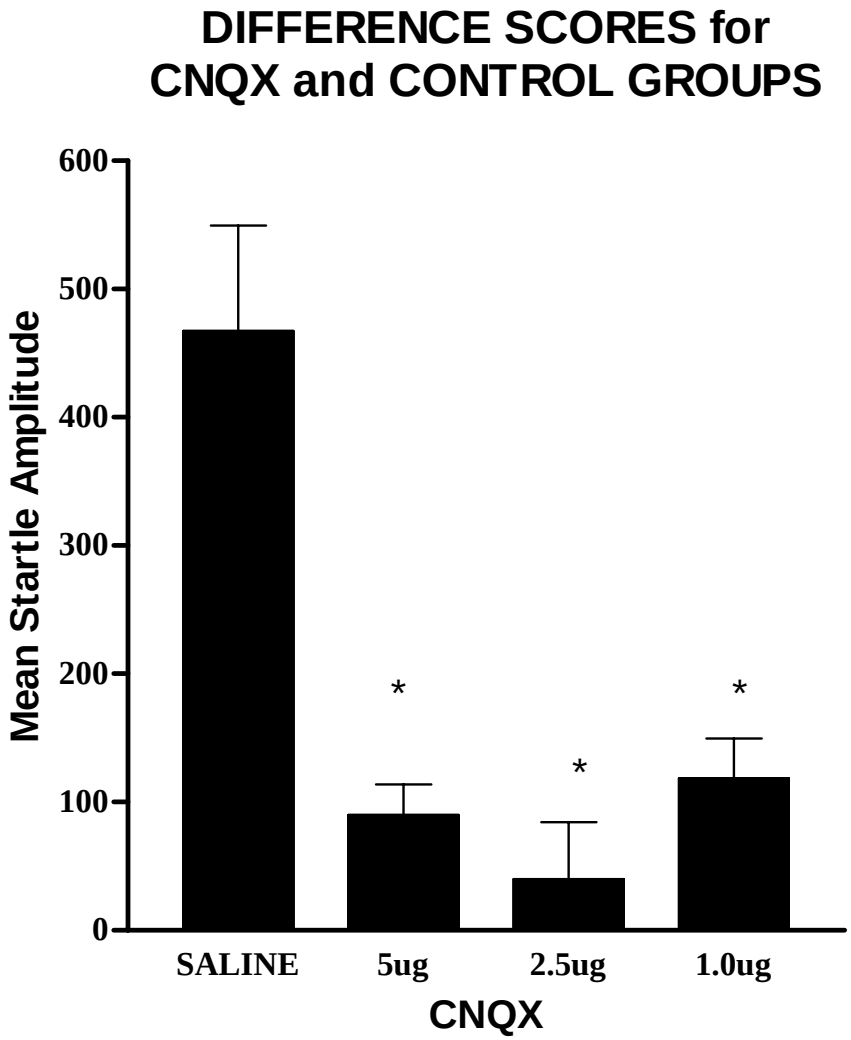

Figure 7. Mean difference scores (light + noise – noise alone) following infusion of saline (N = 8) or CNQX 5ug (N = 8), CNQX 2.5ug (N = 6), and CNQX 1.0ug (N = 7) into the VTA.

4.4 Shock Reactivity

In order to demonstrate that drug infusions did not significantly attenuate the shock reactivity of the subjects a one-way ANOVA was used. There was no significant difference in shock reactivity between the CNQX drug groups and the saline group, $F(2,26) = 0.88$, $p<0.43$, ns. Therefore, the anxiolytic actions of CNQX infused into the VTA cannot be effected by possible drug effects on sensorimotor responding as a suppression of movement amplitude was not induced by the AP5 infusion (see Figure 8).

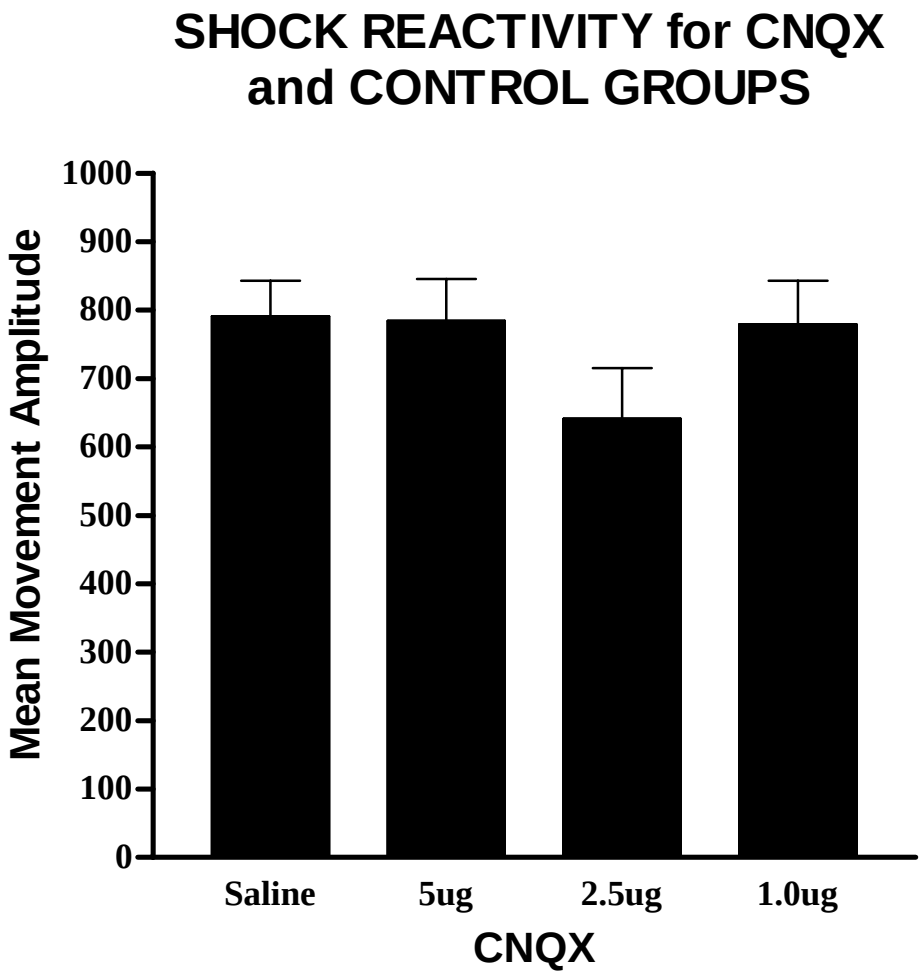

Figure 8. Mean movement amplitude recorded 100ms before shock and 100ms after shock onset following infusion of saline (N = 8) or CNQX 5ug (N = 8), CNQX 2.5ug (N = 6), and CNQX 1.0ug (N = 7) into the VTA. ANOVA did not reveal any significant difference between the groups.

5.0 GENERAL DISCUSSION

5.1 Main Findings

5.1.1 AP5

Intra-VTA infusion of 5ug, 2.5ug and 1.0ug of the NMDA receptor antagonist AP5 significantly blocked fear potentiated startle relative to the control group administered saline. The ANOVA supported the assertion that the saline administered control group displayed an increased fear potentiated startle response when compared to the drug groups administered 5ug, 2.5ug and 1.0ug of AP5 (Figures 2 & 3). Between groups ANOVA was carried out to determine whether there were any variations in shock reactivity post drug administration the results indicated that the three groups that received intra-VTA infusion of AP5 displayed a reaction to shock comparable to the control group (Figure 4). This indicates that the drug did not impair the animal's ability to experience the shock as aversive and respond appropriately. This data supports many assumptions made with regards to VTA DA neurons and their involvement within the neurocircuitry mediating fear conditioning (Greba, Munro, Kokkinidis, 2000). The results of the experiment also provide new insights into the role of the excitatory amino acid (EAA) Glutamate and its possible involvement in the neural processes modulating the changes that govern learning through synaptic plasticity.

Aside from VTA containing neurons, the possibility exists that the infusion of the NMDA receptor antagonist AP5 exerted its affects upon neurons contained within neighbouring anatomical regions. The Substantia Nigra (SN) is one region neighbouring the VTA which could be affected by the spread of intracranial administration of the drug. There is however substantial evidence which has demonstrated functional differences between the VTA and SN. For example, Deutch et al., (1985) demonstrated that stimuli conditioned to be feared have the effect upon presentation of increasing extracellular VTA DA release. This effect has been associated with an enhancement in VTA DA neural activity. Also in support are findings that lesioning the VTA blocks the expression of FPS, whereas 6-OHDA lesions made directly to the SN, which has been shown to reduce DA upon application, does not block the FPS response (Borowski & Kokkinidis, 1996; Hitchcock & Davis 1991). Next, it has been demonstrated that the stimulation of the SN electrically, does not increase acoustic startle amplitudes in the rat. In contrast to this electrical stimulation of the VTA does produce an increase in the rat acoustic startle response (Borowski & Kokkinidis, 1996).

As previous research into this area has noted (Giftkins, Greba, & Kokkinidis, 2002), the primary brainstem startle pathway which runs to the amygdala transverses both the VTA and SN. The placement of the guide cannulas inserted intracranially into the VTA is located dorsal to the startle pathway, however the possibility remains that this circuitry may have become

damaged. Evidence against this occurring is provided however by the results obtained from the control group. The control group subjects were administered saline, and then tested in circumstances paralleling the groups that received the antagonist drugs. Drug and saline infusion was performed, the results demonstrate that shock reactivity levels were not impaired by the drug administration.

The administration if bicuculline, a GABAa receptor antagonist into the VTA increases species typical fear responses such as defensive freezing and the startle response, whereas bicuculline infusion to the SN produces behaviours not associated with emotionality, such as an increase in grooming, head turning, and circling behaviours (Greba et al., 2000; Stevens, Wilson, & Foote, 1974). Finally, it has been shown that lesioning of the VTA or the amygdala produces a decrease in fear responding; whereas lesioning to the SN does not reduce fear responses (Galey, Simon, & LeMoal, 1977).

5.2 CNQX

CNQX is an AMPA receptor antagonist that reportedly binds with AMPA receptors on VTA DA neurons (Harris, Winner, Byrne, & Aston Jones, 2004). The findings from the groups administered 5ug, 2.5ug, and 1.0ug of the AMPA receptor antagonist CNQX provide support for glutamate playing a role in the neurochemistry contributing to the development of CS-UCS associations (Figures 6 & 7). This finding lends further weight to the assertion that glutamatergic AMPA receptors are involved in fear conditioning. As was the case with the subjects administered AP5, those rats administered decreasing doses of CNQX demonstrated normal levels of sensory-motor processing post-drug infusion (Figure 8). Similarly, the levels of reactivity to footshock was also unimpaired as a result of AMPA receptor antagonist administration, this indicates that the animal's perception of the footshock was not impaired.

5.3 Mesocorticolimbic Involvement in Fear Conditioning

Pharmacological and lesion experiments have demonstrated that VTA DA neural activity is a necessary component of the pathway that governs classically conditioned fear (Borowski & Kokkinidis, 1996; Munro & Kokkinidis, 1997; Greba et al., 2000). Repeating the AP5 results, CNQX blocked the ability of a CS – US associations to become formed and as a consequence elicit the fear potentiated startle response upon testing. The results of the CNQX study suggest that AMPA receptors may take part in the complex neural processes necessary for fear

learning and the FPS response. The data from the CNQX experiments also suggests that the VTA may be an area where NMDA and AMPA mediated processes associated with LTP occur. As has been discussed above, the activation of NMDA and AMPA receptors are involved in the cellular processes governing the synaptic alterations associated with LTP. In this study intra-VTA infusion of the AMPA receptor antagonist CNQX had similar effects on learning processes required in fear conditioning as that produced by research into amygdaloid LTP (Sigurdsson, Doyère, Cain, & LeDoux, 2006).

As was the case in past experiments examining the anatgaonism of VTA NMDA receptors within the basolateral nucleus of the amygdala produced a disruption in fear learning (Walker et al., 2002). Given their anatomical and neurochemical connectivity, the results from this study and research into glutamate transmission within the amygdala (Walker et al., 2002) suggest that both areas may participate in the development of fear learning.

5.4 Long Term Potentiation

It has been shown that glutamatergic receptors on VTA DA neurons are involved in drug induced LTP (Harris et al., 2004). This indicates that the processing of information governing conditioned responding may be mediated by glutamatergic inputs into the VTA. Evidence supporting this assertion comes from research investigating LTP-like synaptic enhancements in other brain areas.

Data has shown that the stimulation of hippocampal and amygdala afferents produces synaptic changes significantly associated with LTP (Chapman, Kairiss, Keenan, & Brown, 1990; Walker & Davis, 2002). Davis and colleagues have suggested that the synaptic mechanism involved the production of LTP may also be involved and analogous to the processes involved in fear conditioning (Walker et al., 2002).

It has been suggested that the mechanisms underlying LTP are similar to those processes involved in fear production and the rewarding properties of drug related effects (Rogan, Staubli, & LeDoux, 1997). Further evidence that supports glutamate's role in mediating behavioural conditioning comes from research involving a drug induced form of LTP. Research has demonstrated that Glutamate associated neural plasticity within the VTA is involved in learning to associate specific environmental stimuli that have been temporally linked to exposure from cocaine and morphine exposure (Harris & Aston Jones, 2003; Harris, Winner, Byrne, & Aston Jones, 2004). This research demonstrated that environmental stimuli previously associated with the reinforcing effects of drugs, namely cocaine and morphine, may develop their conditioned association through a glutamatergic process that governs neural plasticity (Harris et al., 2003). The development of conditioned associations between environmental stimuli and drugs of abuse has been shown to involve glutamate based neural plasticity (Winder, Egli, Schramm, Matthews, 2002), it may also be possible that

this same process underlies the formation of conditioned CS – US association in the VTA.

DA modulation and transmission within the mesocorticolimbic systems has been shown to be involved in learning and executing conditioned defensive behaviours (Beninger & Phillips, 1980; Harris et al., 2004). Opiate interaction within VTA DA neurons has been shown to augment DA release through a reduction in inhibitory GABA which typically suppresses DA release (Harris et al., 2004; Johnson & North, 1992). It has been suggested that conditioned environment stimuli act directly on VTA DA cells via excitatory Glutamatergic inputs (Harris et al., 2004; Schultz, 1998; Bespalov, Zvartau, Balster, & Beardsley, 2000). NMDA receptors located within the VTA and nucleus accumbens have been found to play a prominent role in the acquisition and expression of conditioned place preferences for drugs of abuse (Popik & Kolasiewicz, 1999). For example, the administration of the NMDA antagonists AP5 blocked both the acquisition and expression of a conditioned morphine place preference (Tzschentke & Schmidt, 1997). This research demonstrates further the importance of glutamatergic transmission and its role in learning and the expression of conditioned stimulus associations.

5.5 Experimental Considerations

As was the case in studies examining the hippocampus (Chapman et al., 1990), data from this study suggests that glutamatergic NMDA and AMPA receptors antagonists on DA neurons within the VTA, may be involved in the induction of LTP. A statistical ANOVA of shock reactivity between the drug and saline groups (AP5 and CNQX) (Figures 4&8) demonstrated that there were no differences in their mean movement amplitudes. This is important because it indicates that any impairment in fear potentiated startle produced by infusion of both drugs was not due to irreversible damage to the VTA that would be expected to produce hypo-emotivity or decreased fear responding.

It has been reported that administration of the glutamate antagonists drugs AP5 and CNQX have rewarding, non-aversive effects (Harris et al., 2004; David, Durkin, & Cazala, 1998). This suggests that it is unlikely that glutamate antagonists blocked fear learning by reducing the rewarding properties inherent in defensively responding to a CS that predicts footshock. NMDA antagonists administered into the VTA have reportedly produced increases in locomotor activity post infusion also (Cornish, Nakamura, Kalivas, 2001). It has been suggested that the neural actions which enhance locomotor activity require inhibition of VTA GABA cells which are known to project to forebrain regions such as the nucleus accumbens (Van Bockstaele & Pickel, 1995) and regions of the pre-frontal cortex (Carr & Sesack, 2000), which are known to play a role in reward related learning (Wise, 2002). It has been postulated that the reinforcing

effects of glutamate antagonists on cells may in fact be under control of one or more of the areas that receives projection from the VTA (Harris et al., 2004). Considering the rewarding effects produced by NMDA and AMPA receptor antagonist drugs, the administration of these agents would enhance defensive startle behaviours that promote survival (Harris et al., 2004). Conversely, this study demonstrates that these glutamate antagonists administrated to the VTA impair the processes involved in fear conditioning and do not increase the rewarding or behavioural aspects of an acquired fear response. It is therefore hypothesised that the glutamate antagonists AP5 and CNQX disrupt LTP by interfering with the synaptic modifications within the VTA that are required in the acquisition of fear conditioned associations.

Previous research has demonstrated that the administration of methylscopolamine, a non-specific muscarinic receptor antagonist impaired the acquisition of conditioned fear to a CS (Greba et al., 2000). The present findings support the assertion made by Greba and colleagues (2000) who suggested EAA involvement in promoting fear motivation through VTA DA neurons. The results of their study indicate that glutamate receptors in the VTA are required for the acquisition or conditioning of fear potentiated startle responding to a previously neutral CS (Greba et al., 2000). Findings from this research that administration of the NMDA receptor antagonist AP5 blocked the acquisition of FPS is analogous to previous results reporting on observations from hippocampal (Maren & Fanselow, 1995) and amygdala studies (Melia, Miserendino, Sananes, & Davis,

1990). Similar results implicating NMDA receptor antagonism and its involvement in fear conditioning comes from research into drugs of abuse (Harris et al., 2003; 2004). Glutamate receptor antagonism via application of the drugs AP5 and CNQX have also been shown to block the acquisition of a conditioned place preference to drugs such as morphine and cocaine (Harris & Aston Jones, 2003; Harris, Winner, Byrne, & Aston Jones, 2004).

The neural circuitry within the VTA where glutamate antagonists exert their effects on NMDA and AMPA receptors are complex and influenced by a variety of neural systems. The possibility exists that neurons within the VTA require the activation of NMDA and AMPA receptors to promote the synaptic changes associated with LTP (Greba et al., 2000). The results show that inhibition of NMDA receptor function impairs the acquisition of conditioned fear as measured by the FPS response. The same result also held true when the AMPA receptor was antagonised via administration of the antagonist CNQX. These results suggest that NMDA and AMPA receptors in the VTA may facilitate the acquisition of conditioned fear. This suggests that neural circuitry within the VTA undergoes synaptic transformations that have been found to be involved in LTP. This form of neural plasticity involves a variety of chemical interactions at synaptic and receptor sites involving glutamatergic NMDA and AMPA receptor activation.

Evidence exists that supports the involvement of mesolimbic DA neurons arising from the VTA in fear conditioning from the PPTg and the LDTg (Greba et al., 2000).The present study demonstrates again that suppression of mesocorticolimbic DA neurons interferes with the fear potentiated startle response. It is known that neurons within the PPTg are one possible source for EAA's in the VTA (Lavoie & Parent, 1994; Greba et al., 2000). Studies have also demonstrated that application of NMDA and non NMDA receptor agonists produce an increase in VTA DA neuronal activity (Wang & French, 1993; Westerink, Kwint, & De Vries, 1996). These findings raise the question of whether VTA DA neurons are involved in the processes involved in conditioned fear acquisition and memory consolidation.

5.6 Possible Explanations

The hypothesis that excitatory glutamate receptor transmission within that VTA may contribute to the acquisition of fear conditioning in some part through NMDA and AMPA receptor antagonism gains some creditability from this study. Given its connectivity with a number of forebrain regions linked to the processing of emotional material, including the amygdala, hippocampus, thalamus, hypothalamus septum and bed nucleus of the stria terminalis (see Davis, 1992 for review), the VTA may exert its influence over conditioned fear through activation of glutamate receptor systems. Using the FPS response as a measure of conditioned fear, within the VTA it has been possible to begin to investigate

the neural and neurochemical contributors to fear and the development of conditioned associations. It is known that the neurochemistry and neurocircuitry that govern fear conditioning and fear potentiated startle involve complex interactions between a number of different cellular and synaptic processes (Davis, 1993). The results of this study suggest a possible role for glutamatergic NMDA and AMPA receptors within the VTA. The administration of the NMDA receptor antagonist AP5 prior to fear conditioning blocked the acquisition of the FPS response. This indicates that the VTA may be a site where NMDA receptor mediated LTP, is involved in the acquisition of conditioned fear associations. Antagonism of VTA AMPA receptors prior to the fear conditioning procedure also demonstrated that they play a part in pavlovian fear conditioning. The administration of the AMPA receptor antagonist CNQX also blocked the acquisition of a FPS response. This indicates that AMPA receptors within the VTA may also be involved in acquiring a conditioned fear memory.

5.7 Clinical Implications

The present results of this research have demonstrated that the administration of both AP5 and CNQX directly into the VTA blocked FPS. These findings have important implications for the current understanding of the neurochemical basis of disturbances in emotionality associated with abnormal levels of fear and anxiety in psychiatric disorders. Fearful and anxious symptoms common to some psychiatric disorders are often triggered by environmental

stimuli that signal an immediate threat which produce stimulation on circuitry involved in fear and anxiety. If the development of fearful conditioned responses could be blocked prior to new association being formed, this could provide a novel treatment adjunct to existing treatments. The VTA may present one site where future interactions could be directed.

5.8 Conclusion

Data from this study indicates that gutamatergic NMDA and AMPA receptor activation within the VTA is required for the acquisition of learned fear CS – UCS association. It is postulated that the VTA may be a potential site of synaptic plasticity where modifications occur allowing an aversive CS to become associated with a UCS thereby forming a conditioned fear memory. The NMDA and AMPA glutamatergic receptors may also be involved in the same processes that occur in the previously demonstrated utilisation of glutamatergic receptor functioning in the amygdala (Walker et al., 2002). The present results establish the VTA as a site involved in fear motivational responding and indicate that the glutamate receptors NMDA and AMPA may contribute to the induction of LTP in conditioned fear acquisition. In order to further develop an understanding of VTA neural dynamics and those involved in FPS and LTP, future research examining the role of glutamate and its corresponding receptor complex is required. However, study will benefit from data presented by previous research into the VTA and the fear motivational properties of conditioned aversive stimuli.

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